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CD36 as a target to prevent cardiac lipotoxicity and insulin resistance

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ABSTRACT

The fatty acid transporter and scavenger receptor CD36 is increasingly being implicated in the pathogenesis of insulin resistance and its progression towards type 2 diabetes and associated cardiovascular complications. The redistribution of CD36 from intracellular stores to the plasma membrane is one of the earliest changes occurring in the heart during diet induced obesity and insulin resistance. This elicits an increased rate of fatty acid uptake and enhanced incorporation into triacylglycerol stores and lipid intermediates to subsequently interfere with insulin-induced GLUT4 recruitment (i.e., insulin resistance). In the present paper we discuss the potential of CD36 to serve as a target to rectify abnormal myocardial fatty acid uptake rates in cardiac lipotoxic diseases. Two approaches are described: (i) immunochemical inhibition of CD36 present at the sarcolemma and (ii) interference with the subcellular recycling of CD36. Using in vitro model systems of high-fat diet induced insulin resistance, the results indicate the feasibility of using CD36 as a target for adaptation of cardiac metabolic substrate utilization. In conclusion, CD36 deserves further attention as a promising therapeutic target to redirect fatty acid fluxes in the body.

1. Introduction

The healthy heart is characterized by a high rate of (long-chain) fatty acid metabolism. Besides glucose, fatty acids comprise a major substrate for metabolic energy production continuously needed to sustain myocardial contraction [1]. Because under normal conditions the heart stores only small amounts of fat, there is a constant need to extract these fatty acids from plasma. As a result, in the heart there is a fine-tuning of high rates of myocellular fatty acid uptake and of mitochondrial β-oxidation. When this dynamic interplay between fatty acid uptake and metabolism is disturbed, this will result in cardiac pathology. For instance, when the rate of fatty acid delivery to the heart is increased such as during high-fat feeding, the rate of fatty acid oxidation will increase and fatty acids become the predominant substrate, but eventually the uptake will still exceed the rate of oxidation so that fatty acids will be stored intracellularly into triacylglycerols. Likewise, when fatty acid oxidation is impaired such as in the case of carnitine deficiency or inborn errors of fatty acid β-oxidation [2], only part of the incoming fatty acids can enter the oxidation pathway and a major portion is esterified into triacylglycerols. When such lipid storage becomes excessive it is associated with impaired cardiac functioning [3]. This latter condition is referred to as cardiac lipotoxicity [4].

In recent years our understanding of the regulation of cellular fatty acid uptake has markedly increased. Particularly, fatty acid uptake was found to occur by a mechanism that resembles that of cellular glucose uptake reviewed in [5]. In response to an external trigger such as insulin (on adipocytes, heart and skeletal muscle) or to (an increase in) muscular contraction (of heart and skeletal muscle), specific fatty acid transporters translocate from intracellular stores to the plasma membrane to facilitate fatty acid uptake, just as these same stimuli recruit glucose transporters to increase cellular glucose uptake. In the heart, the main fatty acid transporter that functions by this mechanism is the membrane protein CD36. Fatty acid transporters, especially CD36, have been implicated in the pathogenesis of diseases involving alterations in cellular lipid metabolism, including cardiac lipotoxicity. Consequently, fatty acid transporters may serve as molecular target to rectify abnormal tissue fatty acid handling. In the present paper we will explore the possibility of selective targeting of CD36 as therapeutic intervention.

2. Pivotal role of CD36 in myocardial fatty acid uptake

CD36 is a 472-amino acid, heavily glycosylated transmembrane protein (88 kDa) that is well established to facilitate the uptake of (long-chain) fatty acids into adipocytes, heart, and

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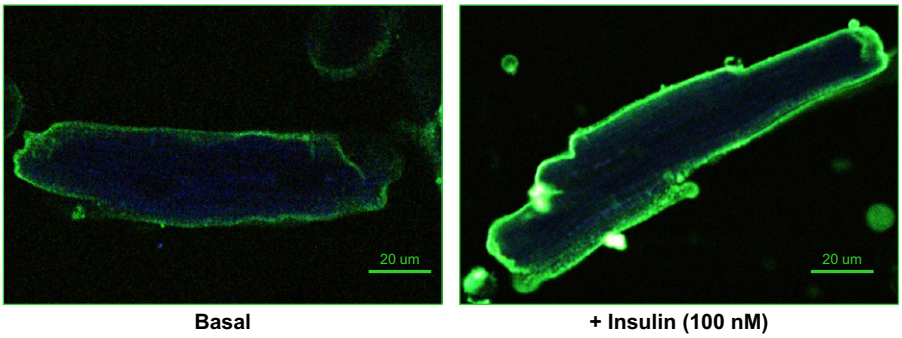

Fig. 1. Two-photon imaging of sarcolemmal CD36 in control cardiomyocytes (*left*) and after insulin treatment (*right*). Cardiomyocytes were isolated from adult male Lewis rats and short-term (15 min) treated with or without 100 nM insulin. Cells then were incubated with mouse anti-mouse CD36 mAb (clone 63), which is known to cross-react with rat CD36, and FITC labeled rabbit–anti–mouse IgA secondary antibodies. Subsequent imaging of the cardiomyocytes was done using the Leica SP5 imaging platform in two-photon mode (Leica Microsystems, Wetzlar, Germany) with the emission filters optimized for FITC detection. The image shown is representative for three observations.

skeletal muscle and, therefore, is also referred to as fatty acid translocase (FAT) [5–7]. CD36 is also known from its function in platelets (thrombospondin binding), in macrophages (scavenger receptor for oxidized LDL), and as gustatory lipid sensor reviewed in [8]. Studies in CD36 null mice have revealed that CD36 contributes for approximately 70% to fatty acid uptake into contracting cardiac myocytes [9]. The importance of CD36 also follows from the observation that mutations in the CD36 gene may markedly affect myocardial fatty acid uptake. For instance, Tanaka et al. [10] reported a patient with two mutations in the CD36 gene that caused a virtual absence of fatty acid uptake as monitored using ^{123}I-BMIPP and SPECT imaging.

A role for CD36 in the regulation of cardiac fatty acid uptake was disclosed when it was found that about 50% of the cellular amount of CD36 is stored in intracellular compartments from where it can be recruited to the sarcolemma to increase the rate of fatty acid uptake [11]. CD36 translocation to the sarcolemma is a rapid and reversible process (Fig. 1) [12], and is triggered by insulin, muscle contraction, and pharmacological agents such as caffeine [13], AICAR, phenylephrine, and okadaic acid (Luiken, JJFP, unpublished observation). At the extracellular site CD36 shows protein–protein interaction with plasmamembrane fatty acid-binding protein (FABPpm), a peripheral membrane protein with a ubiquitous tissue occurrence [14], and at the intracellular site with cytoplasmic FABP that will bind the incoming fatty acids and facilitate their transport to sites of utilization [5,15,16]. Finally, the heart also expresses three members of the family of fatty acid-transport proteins (FATPs), i.e., FATP1, FATP4, and FATP6, but these proteins merely function in the uptake of very long-chain fatty acids (chain length >22) which by action of the synthetase activity of the FATPs are converted directly into very long-chain acyl-CoA esters [17]. Moreover, the FATPs have been shown not to be regulated by reversible translocation, at least not in the heart [18].

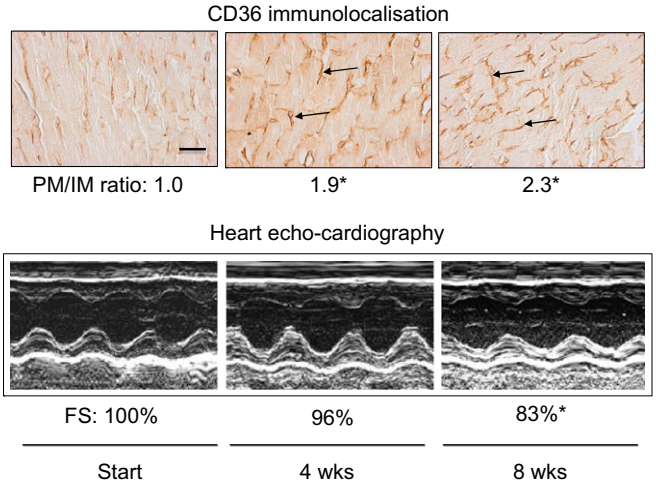

Fig. 2. High-fat diet induces CD36 redistribution to the sarcolemma and left ventricular dysfunction in rats. Adult male Wistar rats received a high-fat diet (50 en% fat) for 8 weeks. *Top panel:* CD36 localization was studied in sections from paraffin-embedded left ventricular tissue obtained from animals sacrificed at the start and after 4 and 8 weeks dietary intervention. Immunohistochemical staining of CD36 was performed with the anti-CD36 monoclonal antibody MO25 and visualized with peroxidase reaction. From these images the ratio of CD36 presence at the plasmamembrane (PM) to that at intracellular membranes (IM) was calculated. Arrows indicate intercalated disks. Scale bar indicates 25 μM. Note that Western blot analysis revealed that total CD36 protein content did not change (data not shown). *Lower panel:* Echocardiography was performed and fractional shortening (FS) calculated at the start and after 4 and 8 weeks on high-fat diet (the value at the start was set at 100%). Representative images are shown. Data obtained from [19].

3. CD36 in cardiac metabolic disease

Various studies have shown the involvement of CD36 in the increased cardiac fatty acid utilization seen in obesity and type 2 diabetes. When rats were subjected to a high-fat diet (50 en%) during 8 weeks, both fatty acid uptake and fatty acid esterification into triacylglycerols measured in isolated cardiomyocytes were increased 1.4–fold when compared to rats fed a low-fat diet (16 en% fat). Total tissue CD36 content was unchanged; however CD36 was relocated to the sarcolemma (2-fold increase; Fig. 2). Cardiac function monitored in vivo by echocardiography revealed a decreased fractional shortening and a lower ejection fraction, indicating cardiac contractile dysfunction (Fig. 2). Interestingly, the increase in sarcolemmal CD36 was seen already after 4 weeks on high-fat diet, when contractile function had not yet changed. Therefore, these combined observations led to the conclusion that exposure to a high-fat diet first induces a relocation of CD36 to the sarcolemma and an associated increase in cardiac fatty acid uptake, and subsequently leads to cardiac contractile dysfunction [19].

Similar observations have been made in human studies which, understandably, have focused on the more accessible skeletal muscle. Bonen et al. [20] showed that in human obesity and type 2 diabetes the increased rate of fatty acid transport into skeletal muscle was associated with an increase in sarcolemmal CD36. Furthermore, in obesity the rate of fatty acid esterification was increased while concomitantly the rate of fatty acid oxidation was not altered, suggesting that the increased rate of CD36-mediated fatty acid transport may contribute to the increased rate of

triacylglycerol accumulation. Indeed, among lean, overweight, obese, and type 2 diabetic individuals, muscle triacylglycerol content was positively correlated with the sarcolemmal CD36 content [20]. Subsequent studies in obese patients by Aguer et al. [21] also revealed that intramyocellular lipid accumulation is related to sarcolemmal CD36 relocation.

The above observations suggest that CD36 can be regarded as a key factor in the etiology of obesity-induced insulin resistance and type 2 diabetes. Our current understanding is that early in the development of insulin resistance alterations occur in the signaling cascades and/or trafficking proteins specifically dedicated to subcellular CD36 recycling, which would result in a selective and permanent relocation of CD36 to the sarcolemma. Importantly, at this stage there would be no concurrent change in the subcellular distribution of the glucose transporter GLUT4. The increased sarcolemmal CD36 abundance, together with an increased plasma fatty acid concentration, would elicit an increased rate of fatty acid uptake and esterification into triacylglycerols, and increased concentrations of fatty acid metabolites such as diacylglycerols and ceramides, as has been shown [22]. The latter compounds will then interfere with insulin-induced GLUT4 translocation to the sarcolemma so that GLUT4 is retained intracellularly and the rate of glucose uptake is lowered, i.e., insulin resistance (Fig. 3). This scenario would provide a mechanism for the observation that during the development of obesity-induced insulin resistance and its progression towards type 2 diabetes and diabetic cardiomyopathy, changes in fatty acid metabolism precede a change in glucose uptake.

4. CD36 as target of metabolic modulation

Based on the key role of CD36 in the early metabolic changes occurring in cardiac myocytes during the development of insulin resistance and type 2 diabetes, it is hypothesized that CD36 may serve as a therapeutic target for the prevention and/or treatment of insulin resistance and diabetes related cardiac contractile dysfunction. Specifically, the intervention would be aimed at limiting CD36-mediated cardiac fatty acid uptake, which in turn would normalize the rate of intracellular fatty acid esterification into triacylglycerols, thereby lowering intramyocardial lipid storage and excess production of fatty acid metabolites. The latter would relieve their inhibitory action on insulin-induced GLUT4 translocation and normalize cardiac glucose uptake and utilization.

A number of experimental studies with transgenic mouse models have indicated the feasibility of this concept. Yang et al. [23] studied mice with a cardiac-restricted overexpression of peroxisome proliferator-activated receptor α (PPARα) which results in a twofold increased myocardial lipid accumulation and left ventricular systolic dysfunction. When these mice were cross-bred with CD36 null animals, both lipid accumulation and systolic function were normalized, suggesting that the absence of CD36 rescues cardiac dysfunction in a genetic model of cardiac lipotoxicity [23]. A physiologically more relevant study was performed by Steinbusch et al. [24]. Mice subjected to a Western-type (high-fat and high-sucrose) diet for 10 weeks showed an 1.8-fold increased cardiac lipid content but normal cardiac contractile function (Fig. 4, left panel). When these mice subsequently underwent surgery for transverse aortic constriction (TAC), which elicits high blood pressure, they developed cardiac hypertrophy and dysfunction, indicating that a mechanical stress (TAC) on top of a metabolic stress (increased lipid metabolism) induces cardiac dysfunction and remodeling. When these interventions were made on CD36 null mice, cardiac lipid accumulation was prevented and TAC-induced cardiac functional and structural changes remained absent (Fig. 4, right panel). Together, these data indicate that CD36 ablation prevents the metabolic stress resulting from a Western-type diet, so that the heart can better cope with the mechanophysical stress that results from high blood pressure [24]. In a broader context, this study also provides evidence that metabolic conditions are a critical factor for the compromised heart and thus warrant further attention.

5. Approaches to target CD36

In principle, targeting of CD36-mediated fatty acid uptake could involve either (i) the functioning of CD36 present at the sarcolemma or (ii) the subcellular recycling of CD36 so as to limit its presence at the sarcolemma. Both approaches will be discussed.

5.1. Inhibition of sarcolemmal CD36

Sulfo-N-succinimidyl esters of long-chain fatty acids are well-known inhibitors of the fatty acid transport function of CD36 since these compounds were used for the disclosure of this function for CD36 [6]. For instance, sulfo-N-succinimidyl oleate (SSO) effectively and completely blocks CD36-mediated fatty acid

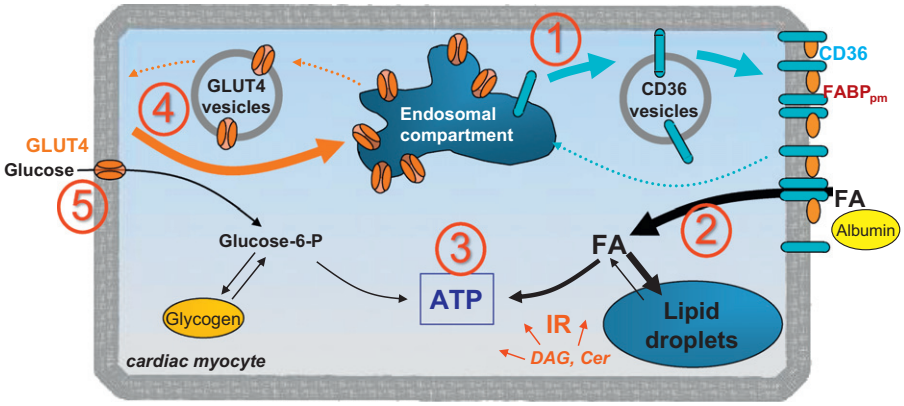

Fig. 3. Juxtaposition of the substrate transporters CD36 and GLUT4 in insulin-resistant muscle. Schematic presentation of a hypothetical model for the development of an impaired GLUT4 translocation in the (pre)diabetic state. While in healthy conditions, both CD36 and GLUT4 are about equally distributed between endosomes and the sarcolemma, in the (pre)diabetic state, there is a shift in CD36 localization from the endosomes to the sarcolemma (step 1) resulting in enhanced fatty acid uptake and storage of fatty acids into triacylglycerols (TAGs; step 2). Fatty acids then become the major substrate for energy production (step 3). Subsequently, fatty acid metabolites such as diacylglyceroles and ceramides inhibit insulin signaling and translocation of GLUT4 from endosomes to the sarcolemma is impaired (step 4), resulting in lowered glucose uptake and decreased incorporation into glycogen (step 5). At that stage, the muscle has become insulin resistant. Adapted from [5], with permission. FA, long-chain fatty acid; DAG, diacylglyceroles; cer, ceramides; IR, insulin resistance.

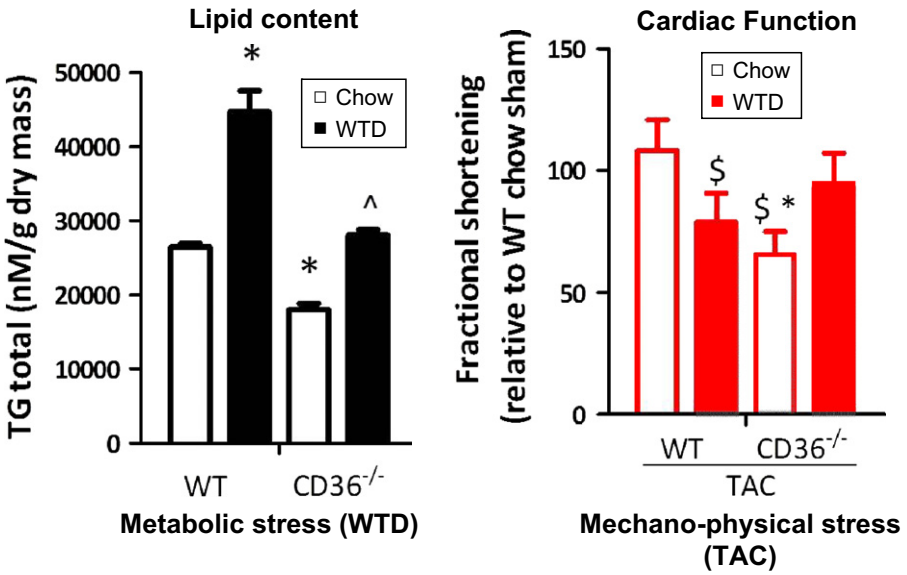

Fig. 4. Absence of CD36 protects against Western-type diet-induced cardiac lipid accumulation (*left*) and cardiac dysfunction following pressure overload (*right*) in mice. Wild-type (WT) and CD36 null ($CD36^{-}$) mice were fed either standard rodent diet (chow) or a Western-type (high fat and high sucrose) diet (WTD) for 10 weeks. Thereafter, mice either were sham operated or underwent transverse aortic constriction (TAC) to induce pressure overload. Diet exposure was continued. Six weeks after surgery, cardiac function (left ventricle fractional shortening) was determined by echocardiography, whereafter the mice were sacrificed for a.o. biochemical determination of cardiac lipid content. Data obtained from [24]. Statistical analysis was done by 2-way ANOVA (with $P < 0.05$ considered significant): * vs. WT on chow diet, ^vs. $CD36^{-/-}$ on chow diet, $ vs. WT/TAC on chow diet.

uptake into cardiomyocytes [9,25,26]. Unfortunately, sulfo-N-succinimidyl esters cannot be used in long-term experiments or in vivo because of their chemical instability [26]. Other ligands that have been described to influence CD36 function include the hexapeptides hexarelin and EP80317. Hexarelin, a synthetic growth hormone releasing (hexa)peptide (GHRP), was shown to exert its cardiovascular activity (increase in coronary perfusion pressure in isolated perfused hearts) by binding to CD36 in a dose dependent manner [27]. The closely related synthetic hexapeptide EP80317 has been described to reduce atherosclerotic lesions in ApoE null mice in a CD36 dependent manner [28]. However, both hexarelin and EP80317 (up to 100 μM) do not affect the rate of fatty acid uptake by isolated rat cardiomyocytes (Angin Y, Steinbusch LKM, unpublished observations). As a result, at present no chemical compounds are known that are suitable for long-term inhibition of the role of CD36 in facilitating cellular fatty acid uptake.

Specific antibodies raised against CD36 are commonly used for immunolocalization studies and Western blotting analyses. Selected antibodies also block CD36 function [29]. However, interestingly, antibodies selected for their inhibition of thrombospondin binding did not affect the fatty acid transport function of CD36 (Luiken JJFP and Bonen A, unpublished observations). In a recent study we used monoclonal antibody clone 63 (also known as clone CRF D2717) raised against mouse CD36 to study whether antibody inhibition of CD36 prevents lipid accumulation and contractile function in rat cardiomyocyte cultures.

Isolated rat cardiomyocytes were cultured for 48 h in a medium containing a relatively high concentration (200 μM) of palmitate, a condition that is know to elicit insulin resistance [30] and reduce contractile function [31]. Indeed, after 48 h of culture in this high-palmitate containing medium, the rate of basal fatty acid uptake was increased 1.6-fold when compared to cells cultured under control conditions (no added palmitate) and was accompanied by a 4.4-fold increased cellular triacylglycerol content (Fig. 5). Concomitantly, the sarcolemmal presence of CD36 was markedly increased (data not shown). While insulin was able to stimulate fatty acid uptake in control cells (by 1.5-fold), cardiomyocytes cultured in high-palmitate medium did not respond to insulin (Fig. 5), thus reflecting the induction of insulin resistance. Likewise, glucose uptake was affected in a similar manner (data not shown). The same set of experiments were also performed in the presence of anti-CD36 antibodies added to the culture medium at the start of the 48 h incubation. The addition of these antibodies prevented the increase in basal fatty acid uptake and loss of its insulin-responsiveness seen in the presence of high palmitate alone (Fig. 5). Importantly, the antibodies completely prevented the high-palmitate induced increase in triacylglycerol content. Taken together, these data demonstrate that immunochemical inhibition of CD36 prevents elevated fatty acid uptake, lipid accumulation, and possibly contractile dysfunction in cardiomyocytes cultured in a lipotoxic medium.

5.2. Modulation of subcellular CD36 recycling

The presence of CD36 at the sarcolemma is the net result of the rate of CD36 translocation from endosomes to the sarcolemma (exocytosis) and the rate of CD36 internalization towards its intracellular storage compartment (endocytosis). Like with other membrane transporters such as the glucose transporter GLUT4 and lipoprotein receptors, both of these processes are under the control of a large number of proteins that together form the so-called trafficking machinery [32]. One of the families of trafficking proteins involved is that of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). These proteins are present at the transport vesicle (v-SNAREs) and the target membrane (t-SNAREs), and are mediators of membrane fusion [33]. This process is highly selective as a v-SNARE interacts with only a specific subset of t-SNAREs to form a SNARE complex that initiates fusion. For instance, at the plasma membrane the v-SNARE vesicle-associated membrane protein-2 (VAMP2) interacts with the t-SNAREs syntaxin4 and SNAP23 to initiate fusion of GLUT4-containing vesicles with the plasma membrane upon insulin stimulation [34].

We examined the involvement of members of the VAMP protein family in the subcellular trafficking of GLUT4 and CD36

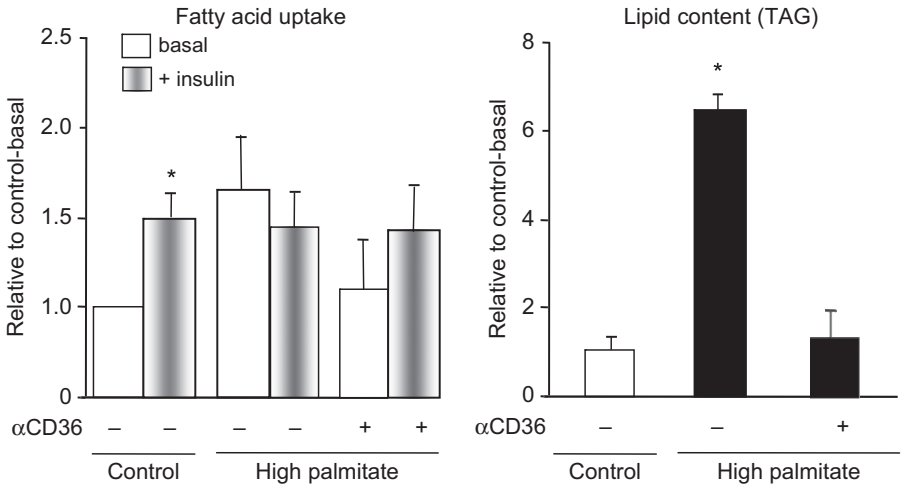

Fig. 5. Immunochemical inhibition of CD36 prevents elevated fatty acid uptake and lipid accumulation in cardiomyocytes cultured under insulin-resistance-inducing conditions. Isolated rat cardiomyocytes were cultured for 48 h either in control medium (containing a.o. 20 μM palmitate, palmitate:albumin 0.3:1) or in a medium containing a high palmitate concentration (200 μM palmitate, palmitate:albumin 3:1) in the absence or presence of anti-CD36 antibodies (CRF D2717). Cells were then washed, allowed to recover for 30 min prior to short-term (15 min) insulin (100 nM) addition and subsequent measurement of ^{14}C-palmitate uptake (measured as described previously [11]). Intramyocellular lipid content (triacylglyceroles, TAG) was determined following lipid extraction and thin-layer chromatography. Values are given as mean ± SEM for $n=5$. Statistical analysis was done by Student's t test (with $P<0.05$ considered significant): * vs. basal.

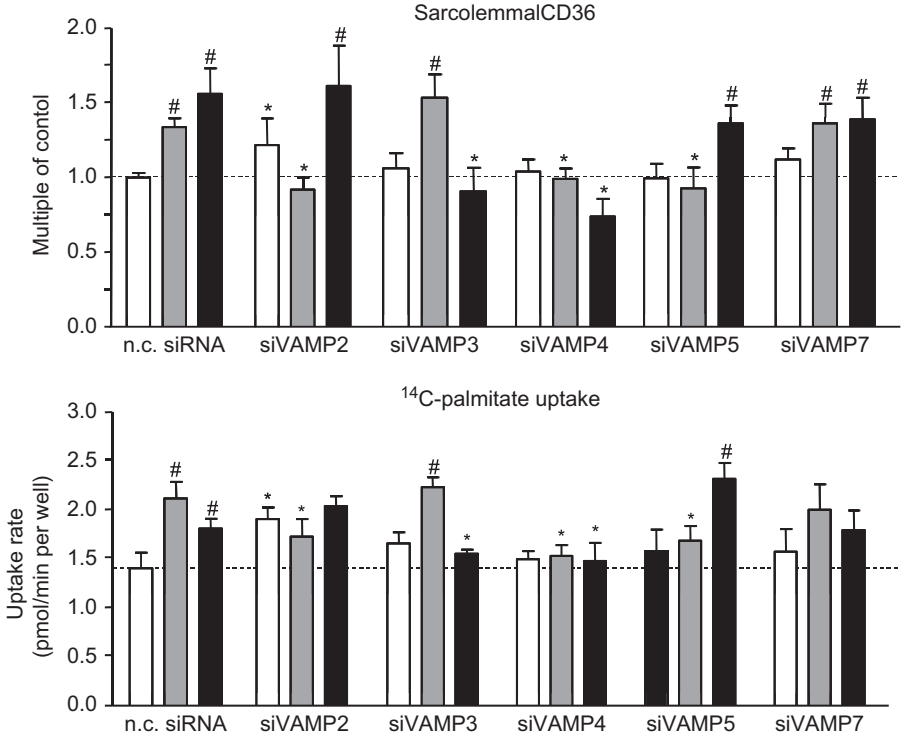

Fig. 6. Distinct vesicle-associated membrane protein (VAMP) isoforms function in insulin- and contraction-induced translocation of CD36 in cultured cardiomyocytes. Genes encoding VAMP isoforms were transiently knocked down using small interfering RNAs (siRNA) in HL-1 atrial cardiomyocytes. Cells were serum depleted prior to short-term (30 min) treatment with insulin (200 nM) or oligomycin (1 μM; increases the AMP/ATP ratio similarly to contraction) whereafter sarcolemmal CD36 was measured by immunostaining, or ^{14}C-palmitate uptake was measured during 10 min incubation. Values are given as mean ± SEM for $n=6$. Statistical analysis was done by Student's t test (with $P<0.05$ considered significant): * vs. corresponding value in control group, ♯ vs. corresponding basic value. Data obtained from [35]. n.c., non-coding.

in cultured cardiomyocytes [35]. Three VAMPs were demonstrated to be required for both GLUT4 and CD36 translocation, either specifically in insulin-induced translocation (VAMP2, VAMP5) or in contraction-induced translocation (VAMP3). In addition, VAMP7 was found to be specifically involved in GLUT4 traffic (mediating basal GLUT4 retention) and VAMP4 specifically in CD36 traffic (both insulin- and contraction-induced translocation) [35]. Fig. 6 (upper panel) shows the plasmalemmal abundance of CD36 after knock-down of the genes encoding for the various VAMP isoforms, both under basal conditions (white bars) and after stimulation by insulin (gray bars) or contraction (black bars). The changes in CD36 presence at the sarcolemma correlate with concomitant changes in the rate of fatty acid uptake by the transfected cells (Fig. 6, lower panel). These observations indicate the possibility to use VAMPs to discriminate between GLUT4 and CD36 translocation, and thus separately modulate cardiac glucose and fatty acid uptake.

In follow-up studies we plan to test this latter concept by manipulating the presence of selective VAMPs in cardiomyocytes and then monitor the vulnerability of the cells for lipotoxic conditions. For instance, we hypothesize that overexpression of VAMP3 might prevent the development of insulin resistance in these cells because this VAMP isoform is specifically required for contraction-induced transporter translocation, and VAMP3 overexpression may thus mimick the effects observed after acute exercise.

6. Concluding remarks

Abnormal rates of fatty acid uptake and/or utilization by the heart are seen under a number of conditions, and are often associated with cardiac disease. In this paper we focused on a mismatch between fatty acid uptake and oxidation which leads to an excessive intracellular storage of fatty acids into di- and triacylglycerols and subsequent cardiac dysfunction, a condition referred to as cardiac lipotoxicity. Myocardial fatty acid uptake is largely regulated by the membrane fatty acid transporter CD36, especially through its continuous recycling from intracellular stores to the sarcolemma triggered by a.o. insulin and myocyte contractions. In view of this pivotal role of CD36 in governing myocardial fatty acid uptake, it was hypothesized that manipulating CD36 will affect the rate of fatty acid uptake and thus may serve as a useful therapeutic intervention to restore abnormalities in the matching of cellular fatty acid uptake to oxidation. Both inhibition of sarcolemmal CD36 and manipulation of the subcellular trafficking of CD36 were shown in in vitro model systems to be effective approaches to normalize aberrant fatty acid handling and features of cardiolipotoxicity seen under lipotoxic conditions. As a result, selective targeting of CD36 holds promise as therapeutic intervention. Future studies with more complex model systems (e.g., intact perfused heart) and in vivo experimental animals should be performed to examine whether this proof-of-concept may be extrapolated to routine medical treatment.

Two approaches were used to affect the role of CD36 in myocardial fatty acid uptake in the in vitro model systems, i.e., immunochemical inhibition and overexpression of one of the trafficking proteins specifically involved in CD36 recycling (when compared to that of GLUT4). Inhibition of CD36 in vivo is expected to be more feasible with specific ligands of small molecular size (< 1 kD) so that these can easily pass through the endothelial clefts and reach the cardiac myocytes. Sulfo-N-succinimidyl-esters of long-chain fatty acids were found not useful because of their chemical instability when in aqueous solution [26]. Compounds that would specifically target selected members of the trafficking proteins involved in CD36 recycling to our knowledge have not been described. Alternatively, selected activation of signaling proteins upstream of the trafficking proteins would also be a theoretical option.

It should be kept in mind that our current understanding of the regulation of CD36 presence and function at the sarcolemma in relation to myocardial fatty acid uptake is only limited. For instance, CD36 is hypothesized to function in specific regions of the sarcolemma, e.g. microdomains like lipid rafts, and caveolae, and does so in interaction with specific partner proteins such as FABPpm and perhaps other (membrane-associated or other) proteins. For instance, for 3T3-L1 adipocytes it was shown that plasma membrane lipid rafts regulate the surface availability of CD36 and thereby control fatty acid uptake [36]. Similarly, the role of the many glycosylation sites of CD36 remains underexplored, although very recently evidence was reported for the importance of O-linked-β-N-acetylglucosamine for the membrane recruitment of CD36 and its role in fatty acid uptake [37]. Finally, CD36 is welldocumented to have two extracellular phosphorylation sites (at Thr-92 and Ser-237) but their significance is still unclear [reviewed in 5]. As a result, it is feasible that the functioning of CD36 as a sarcolemmal fatty acid transporter may also be manipulated by other approaches than described here. The in vitro model systems applied in the present work may be used for screening purposes.

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